

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>C12N 15/00, 15/29, 15/82, A01H 3/00, 4/00, 5/00</b>		<b>A3</b>	(11) International Publication Number: <b>WO 99/66026</b>
			(43) International Publication Date: 23 December 1999 (23.12.99)
(21) International Application Number: <b>PCT/US99/13584</b>		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 15 June 1999 (15.06.99)		<b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(30) Priority Data: 60/089,322 15 June 1998 (15.06.98) US			
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(71)(72) Applicant and Inventor: CHRISTOU, Paul [US/GB]; Norwich Research Park, Colney, Norwich NR4 7UH (GB).			
(72) Inventors: STROGER, Eva; Norwich Research Park, Colney, Norwich NR4 7UH (GB). FISCHER, Rainer; Worringergweg 1, D-52074 Aachen (DE). MARTIN-VAQUERO, Carmen; Norwich Research Park, Colney, Norwich NR4 74H (GB). SCHILLBERG, Stefan; Norwich Research Park, Colney, Norwich NR4 7UH (GB). MA, Julian, K.-C.; UMDS, Guy's Hospital, 28th floor, Guy's Tower, St. Thomas Street, London SE1 9RT (GB).		(88) Date of publication of the international search report: 27 January 2000 (27.01.00)	
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(54) Title: METHODS AND MEANS FOR EXPRESSION OF MAMMALIAN POLYPEPTIDES IN MONOCOTYLEDONOUS PLANTS

Promoter	5'UTR	Leader peptide	Gene of Interest	sig	3'UTR	pA
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## (57) Abstract

Rice, wheat and other monocotyledonous plants are transformed with expression cassettes for production of mammalian polypeptides, such as antibodies. Endoplasmic reticulum (ER) retention signals, 5'untranslated regions and leader peptides are employed in various combinations to provide high expression yield. Multi-chain complexes such as four-chain secretory antibodies are produced by expression of component polypeptides from separate vectors all introduced into the same cell by transformation.

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/13584

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : C12N 15/00, 15/29, 15/82; A01H 3/00, 4/00, 5/00

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 320.1, 419, 440; 536/23.6, 24.1, 23.7; 800/278, 295, 298, 320, 320.1, 320.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, CABA, CAPLUS, GENBANK

search terms: monocot, corn; wheat, immunoglobulin, antibody, immunize, endoplasmic reticulum

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MA et al. Plant Antibodies for Immunotherapy. Plant Physiol. 1995, Vol. 109, pages 341-346, see entire document.	1, 3-5, 7-34
Y	HIEI et al. Efficient transformation of rice (Oryza sativa L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. The Plant Journal. 1994, Vol. 6, No. 2, pages 271-282, see entire document.	1, 3-5, 7-34

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*B* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 17 NOVEMBER 1999	Date of mailing of the international search report 09 DEC 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer THOMAS HAAS Telephone No. (703) 308-0196

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/13584

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☒ Claims Nos.: 2, 6,  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:  
  
Claims 2 and 6 are directed to DNA sequences. The computer readable form of these sequences is defective and as such claims 2 and 6 could not be searched.
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

**INTERNATIONAL SEARCH REPORT**

International application No.  
PCT/US99/13584

**A. CLASSIFICATION OF SUBJECT MATTER:**  
**US CL :**

435/69.1, 320.1, 419, 440; 536/23.6, 24,1, 23.7; 800/278, 295, 298, 320, 320.1, 320,2



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(74) Agent: SCHOFIELD, Mary, Anne; Fulbright &amp; Jaworski L.L.P., 666 Fifth Avenue, New York, NY 10103 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

**Published***Without international search report and to be republished upon receipt of that report.*

(54) Title: METHODS AND MEANS FOR EXPRESSION OF MAMMALIAN POLYPEPTIDES IN MONOCOTYLEDONOUS PLANTS

<b>Promoter</b>	<b>5'UTR</b>	<b>Leader peptide</b>	<b>Gene of interest</b>	<b>sig</b>	<b>3'UTR</b>	<b>pA</b>
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## (57) Abstract

Rice, wheat and other monocotyledonous plants are transformed with expression cassettes for production of mammalian polypeptides, such as antibodies. Endoplasmic reticulum (ER) retention signals, 5'untranslated regions and leader peptides are employed in various combinations to provide high expression yield. Multi-chain complexes such as four-chain secretory antibodies are produced by expression of component polypeptides from separate vectors all introduced into the same cell by transformation.

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**METHODS AND MEANS FOR EXPRESSION OF  
MAMMALIAN POLYPEPTIDES IN MONOCOTYLEDONOUS PLANTS**

The present invention relates to expression of transgenes in  
5 plants, especially monocots, in particular non-plant or  
mammalian genes encoding polypeptides such as antibodies and  
antibody fragments. Expression constructs, transformed  
plants and cells and various methods are provided in  
accordance with various aspects of the invention.

10

Plants offer a number of potential advantages for the  
production of polypeptides of industrial or medical utility,  
such as mammalian proteins, including antibody molecules,  
whether complete antibodies or fragments such as single-chain  
15 Fv antibody molecules (scFv's), and fusion proteins.  
Synthesis of functional antibodies in transgenic plants was  
first demonstrated by Hiatt et al. (Nature (1989) 342: 76-78)  
and subsequently single chain fragments have been  
successfully expressed in leaves of tobacco and *Arabidopsis*  
20 plants (Owen et al. (1992) Bio/Technology 10: 790-794;  
Artsaenko et al. (1995) The Plant J 8: 745-750; Fecker et al.  
(1996) Plant Mol Biol 32: 979-986). Fiedler et al.  
(Bio/Technology (1995) 13: 1090-1093) have shown the  
feasibility of long-term storage of scFv's in tobacco seeds.

Almost exclusively, such work has been in dicotyledonous plants. However, monocot crop plants such as wheat and rice have advantages over dicots such as tobacco in not containing noxious chemicals such as alkaloids. This increases possibilities for safe production of polypeptides for pharmaceutical use. Furthermore, crop plants are of particular significance in food contexts, allowing for provision of "functional foods" which may have potential health benefits. An exemplary application is anti-dental caries antibodies, e.g. as expressed by Ma et al. (Eur J Immunol 24: 131-138 (1994); *Plant Physiology* 109, 341-346 (1995); *Science* (1995) 268, 716-719) in transgenic tobacco (not a functional food as such).

As far as the present inventors are aware the only experimental example of expression of an antibody or other mammalian protein in a monocot is disclosed in WO98/10062 (Monsanto), published 12 March 1998. This document reports expression of antibody light and heavy chains from separate plasmids in transgenic maize plants, under the control of the rice glutelin-1 promoter.

The present inventors have devised various expression constructs for mammalian genes such as antibodies to be produced in transgenic plants, especially monocots, preferably barley, rice, corn, wheat, oat, sorghum, more

preferably wheat, rice. As noted, no-one has previously reported successful expression of such genes in these plants. Experimental evidence described below shows various advantages and benefits from use of different aspects of the expression constructs.

In one aspect of the present invention it has been found that levels of antibody expression in monocots can be enhanced by employing an endoplasmic reticulum (ER) retention signal.

Such a signal is a peptide tag usually including the amino acid sequence Lys Asp Glu Leu (KDEL) (SEQ ID NO. 2) or His Asp Glu Leu (HDEL) (SEQ ID NO. 4). Artsaenko et al. employed KDEL in expression of a single-chain Fv antibody against abscisic acid in the dicot tobacco (*The Plant J.* (1995) 8:745-750), but this has not previously been shown to be functional in monocots.

In another aspect of the present invention, various leader peptide sequences have been found to enhance antibody expression in plants, especially monocots. None of these have previously been shown to be effective in plants. Details are provided below, but no measurable expression of antibody molecule was found in rice calli using a construct without a leader peptide sequence.

25

In a still further aspect of the present invention various 5'

untranslated regions (5'UTR) have been employed in expression of antibody molecules in plants in particular the chalcone synthase and omega 5'UTR's (see below for details). Again, none of these have previously been shown to be effective as  
5 demonstrated herein in plants, especially monocots.

Various aspects of the invention provide nucleic acid constructs and vectors including one or more of these elements, transformed host cells, which may be microbial or  
10 plant, transgenic callus and suspension cultures and plants and various methods for provision or use of such constructs, vectors, host cells, cultures and plants in production of non-plant, particularly eukaryotic polypeptides, such as antibody molecules.

15

*Brief description of the figure*

Figure 1 shows an schematic of the components in expression constructs according to the present invention. In addition  
20 to the promoter and the gene of interest, one or more of the other elements (5'UTR, leader peptide, signal (e.g. KDEL), 3'UTR, pA - polyadenylation signal) may be included and the present invention provides any combination of these elements.

25 In accordance with a first aspect of the present invention there is provided a plant cell or seed, preferably monocot,

containing a polypeptide produced by expression within the cell or seed from an expression cassette including a coding sequence for the polypeptide fused to an endoplasmic reticulum (ER) retention signal.

5

The retention signal may be a peptide with the amino acid sequence KDEL (SEQ ID NO. 2) or HDEL (SEQ ID NO. 4). KDEL may be encoded by the nucleotide sequence AAA GAT GAG CTC (SEQ ID NO. 1) and HDEL may be encoded by CAT GAT GAG CTC (SEQ ID NO. 3). Other sequences encoding the amino acids but differing from these nucleotide sequences by virtue of degeneracy of the genetic code may be employed. The KDEL or HDEL encoding sequence may be operably linked to a coding sequence for the polypeptide to provide a coding sequence for a fusion of the polypeptide and ER retention signal.

15

Generally the retention signal is placed at the C-terminus of the polypeptide. The ER-retention signal may be preceded by a linker sequence, such as (Gly)<sub>4</sub>Ser (SEQ ID NO. 5) and/or Arg Gly Ser Glu (RGSE) (SEQ ID NO. 6) (Wandelt et al. (1992) Plant J. 2(2): 181-192).

20

In accordance with a second aspect of the present invention there is provided a plant cell or seed, preferably monocot, containing a polypeptide produced by expression within the cell or seed from an expression cassette including a coding sequence for the polypeptide and an 5' untranslated leader

25

sequence (5'UTR). The 5'UTR may be that of the chalcone synthase gene of petunia (Reimold et al. (1983) EMBO J 2: 1801-1805) or a modified form including one or more additions, deletions, substitutions or insertions of one of  
5 more nucleotides, preferably modified to include the T's emboldened in the following sequence:

GAATTCACAACACAAATCAGATTTATAGAGAGATTTATAAAAAAAAAAAAAACATATG

(SEQ ID NO. 7). The 5'UTR may be that of the TMV omega gene (Gallie et al. (1992) NAR 20: 4631-4638) or a modified form  
10 including one or more additions, deletions, substitutions or insertions of one of more nucleotides, preferably including modifications as described by Schmitz et al. (1996) NAR 24: 257-263; incorporated herein by reference. The omega untranslated leader sequence from the U1 strain of TMV is (at  
15 the RNA level):

GUAUUUUUACAACAAUUACCAACAACAACAAACAACAAACAACAUUACAUUACUAUUUAC  
AAUUACAATG (SEQ ID NO. 8). (Obviously the "U's" are "T's" at the DNA level. The initiation codon is indicated at the end of the sequence here.) One modification preferred in

20 accordance with embodiments of the present invention is to alter the underlined AUU to AGG. Additionally, one or both of the underlined A's may be deleted.

A preferred modified sequence is:

GUAUUUUUACAACAAUUACCAACAACAACAACAACAACAUUACAUUACUAUUUACAA  
25 GGACCAUGG (SEQ ID NO. 9). In addition to the preferred AUU - > AGG modification, this also includes a near-Kozak sequence

ACCAUGG, where the AUG is the initiation codon.

In accordance with a third aspect of the present invention there is provided a plant cell or seed, preferably monocot, containing a polypeptide produced by expression within the cell or seed from an expression cassette including a coding sequence for the polypeptide and a leader peptide. A leader peptide may be used to direct the product to a particular cellular compartment. The leader peptide may be of mammalian origin, and may be murine, such as an immunoglobulin light or heavy chain leader peptide. The nucleotide sequence used in the construct to encode the leader peptide may be codon optimised for expression in the plant of interest, preferably monocot, e.g. rice or wheat. A preferred leader peptide useful in accordance with this aspect of the present invention is that of the TMV virion specific mAb24 of Voss et al. (Mol Breed (1995) 1: 39-50) (incorporated herein by reference). Modified forms may be employed. As with other elements for use in expression cassettes in accordance with various aspects of the present invention, the coding sequence may be codon optimised for monocot codon usage according to Angenon et al. (FEBS (1990) 271:144-146) (incorporated herein by reference). The leader peptide may be vacuole targeting signal, such as the leader peptide of a strictosidine synthase gene, e.g. that of the *Catharanthus roseus* strictosidine synthase (McKnight et al., Nucleic Acids

Research (1990), 18, 4939; incorporated herein by reference) or of *Rauwolfia serpentina* strictisodine synthase (Kutchan et al. (1988) *FEBS Lett* 237 40-44; incorporated herein by reference). For a review of vacuole targeting sequences see

5 Neuhaus (1996) *Plant Physiol Biochem* 34(2) 217-221. The leader peptide may be a chloroplast targeting signal such as of the pea rubisco leader peptide sequence (Guerineau et al. (1988) *NAR* 16 11 380) (incorporated herein by reference). For a review of chloroplast targeting peptides see van Heijne et

10 al. (*Eur J Biochem* (1989) 180: 535-545) or Kavanagh et al. (MGG (1988) 215: 38-45) or Karlin-Neumann et al. (*EMBO J* (1986) 5: 9-13) (all incorporated herein by reference). The leader peptide may be a 5' sequence of a seed storage protein, dicot or monocot, causing transport into protein

15 bodies, such as the *Vicia fabia* legumin B4 leader (Baeumlein et al. *Mol Gen Genet* (1991) 225: 121-128) (incorporated herein by reference).

One aspect of this invention is a cereal plant cell or seed

20 containing a mammalian protein produced by expression within the cell or seed from an expression cassette comprising a coding sequence for the protein.

In a further aspect, the present invention provides a corn

25 plant cell or seed containing a mammalian protein produced by expression within the cell or seed from an expression



cassette including a coding sequence for the protein.

In a further aspect, the present invention provides a rice plant cell or seed containing a mammalian protein produced by  
5 expression within the cell or seed from an expression cassette including a coding sequence for the protein.

A still further aspect of the invention the present invention provides a wheat plant cell or seed containing a mammalian  
10 protein produced by expression within the cell or seed from an expression cassette including a coding sequence for the protein.

In further aspects of the present invention there are  
15 provided methods for the production of plant cells in accordance with the aspects disclosed above, the methods including introducing into a plant cell nucleic acid including the specified expression construct. Suitable techniques for this, including for vector construction, plant  
20 cell transformation, and plant regeneration are discussed below.

Thus, for example, one of these aspects of the invention provides a method including introducing into a plant cell,  
25 especially monocot, nucleic acid including an expression cassette including a coding sequence for a polypeptide of

interest fused to an endoplasmic reticulum (ER) retention signal. Introduction of nucleic acid into cells may be referred to as "transformation" and resultant cells may be referred to as "transgenic". This is without limitation to  
5 any method or means used to introduce the nucleic acid into the cells.

A transformed cell may be grown or cultured, and further aspects of the present invention provide a suspension culture  
10 or callus culture including such cells. As noted below, further aspects provide plants and parts thereof, and methods of production of plants by transformation of cells and regeneration.

15 It should be noted that plant cells transiently expressing the desired polypeptide following transformation with the appropriate expression cassette are provided by the present invention, but a further aspect provides a method of making a plant cell, preferably monocot, including an expression  
20 cassette as disclosed, the method including:

- (i) introducing a nucleic acid vector suitable for transformation of a plant cell and including the expression cassette into the plant cell, and,
- (ii) causing or allowing recombination between the vector and  
25 the plant cell genome to introduce the expression cassette into the genome.

In a still further aspect the present invention provides a method of making a plant, the method including:

- (i) making plant cells as disclosed; and
- (ii) regenerating a plant from said plant cells or

5 descendants thereof. Such a method may further include cloning or propagating said plant or a descendant thereof containing the relevant expression cassette within its genome.

10 In various embodiments of the present invention the cell or seed is actively producing the polypeptide or protein.

The expressed polypeptide is preferably a eukaryotic, non-plant protein, especially of mammalian origin, and may be  
15 selected from antibody molecules, human serum albumin (Dugaiczky et al. (1982) *PNAS USA* 79: 71-75 (incorporated herein by reference), erythropoietin, other therapeutic molecules or blood substitutes, proteins within enhanced nutritional value, and may be a modified form of any of  
20 these, for instance including one or more insertions, deletions, substitutions and/or additions of one or more amino acids. (The coding sequence is preferably modified to exchange codons that are rare in monocots in accordance with principles for codon usage.)

25

In preferred embodiments of the present invention, a

mammalian protein is an antibody molecule, which includes an polypeptide or polypeptide complex including an immunoglobulin binding domain, whether natural or synthetic. Chimaeric molecules including an immunoglobulin binding domain fused to another polypeptide are therefore included. Example binding fragments are (i) the Fab fragment consisting of VL, VH, CL and CH1 domains; (ii) the Fd fragment consisting of the VH and CH1 domains; (iii) the Fv fragment consisting of the VL and VH domains of a single antibody; (iv) the dAb fragment (Ward, E.S. et al., Nature 341, 544-546 (1989) incorporated herein by reference)) which consists of a VH domain; (v) isolated CDR regions; (vi) F(ab')<sub>2</sub> fragments, a bivalent fragment including two linked Fab fragments (vii) single chain Fv molecules (scFv), wherein a VH domain and a VL domain are linked by a peptide linker which allows the two domains to associate to form an antigen binding site (Bird et al, Science, 242, 423-426, 1988; Huston et al, PNAS USA, 85, 5879-5883, 1988); (viii) bispecific single chain Fv dimers (PCT/US92/09965) and (ix) "diabodies", multivalent or multispecific fragments constructed by gene fusion (WO94/13804; P. Holliger et al Proc. Natl. Acad. Sci. USA 90 6444-6448, 1993) (all incorporated herein by reference). Monospecific but bivalent diabodies can be produced by expression from a single coding sequence, wherein the polypeptides associate to form dimers including two antigen-binding sites. Bispecific diabodies are formed by

association of two different polypeptides, expressed from respective coding sequences.

Where the desired product is a two-chain or multi-chain polypeptide complex (e.g. Fab molecule or bispecific diabody), the expression cassettes may be introduced into plant cells in accordance with the present invention on the same vector or on separate vectors. In one particular aspect of the invention a plant cell, preferably monocot, is transformed separately with four vectors, each including nucleic acid encoding one of the four chains of a secretory antibody, namely the heavy chain, light chain, secretory component and J chain.

The product may be a fusion protein including different proteins or protein domains. For example, certain embodiments of the present invention relate to provision of fusion proteins in which an antibody molecule (such as a scFv molecule or one or both chains of a multimeric antibody molecule such as an Fab fragment or whole antibody) is fused to a non-antibody protein domain, such as interleukin 2, alkaline phosphatase, glucose oxidase (an example of a biological response modifier), green fluorescent protein (an example of a colorimetric label). The non-antibody molecule may be fused to the antibody component at the latter's N- or C-terminus.

Those skilled in the art are well able to construct vectors and design protocols for recombinant gene expression in plants. Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including  
5 promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. For further details see, for example, *Molecular Cloning: a Laboratory Manual*: 2nd edition, Sambrook et al, 1989, Cold Spring Harbor Laboratory Press.

10 Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in *Current Protocols in Molecular*  
15 *Biology*, Second Edition, Ausubel et al. eds., John Wiley & Sons, 1992. The disclosures of Sambrook et al. and Ausubel et al. are incorporated herein by reference. Specific procedures and vectors previously used with wide success upon plants are described by Bevan (Nucl. Acids Res. 12, 8711-8721  
20 (1984)) and Guerineau and Mullineaux (1993) (Plant transformation and expression vectors. In: *Plant Molecular Biology Labfax* (Croy RRD ed) Oxford, BIOS Scientific Publishers, pp 121-148).

25 Selectable genetic markers may be used consisting of chimaeric genes that confer selectable phenotypes such as

resistance to antibiotics such as kanamycin, hygromycin, phosphinotricin, chlorsulfuron, methotrexate, gentamycin, spectinomycin, imidazolinones and glyphosate.

- 5 The vector backbone may be pUC (Yanisch-Perron *et al.* (1985) *Gene* 33: 103-119) or pSS (Voss *et al.* (1995) *Mol Breed* 1: 39-50).

The expression cassette employed in accordance with aspects  
10 of the present invention may include the coding sequence under the control of an externally inducible gene promoter to place expression under the control of the user. A suitable inducible promoter is the GST-II-27 gene promoter which has been shown to be induced by certain chemical compounds which  
15 can be applied to growing plants. The promoter is functional in both monocotyledons and dicotyledons. The GST-II-27 promoter is also suitable for use in a variety of tissues, including roots, leaves, stems and reproductive tissues.

- 20 Other suitable promoters include any constitutive promoter and any seed-specific promoter. Examples include the maize ubiquitin promoter and intron (US-A-5510474), CaMV 35S promoter (Gardner *et al.* (1981) *NAR* 9: 2871-2888), and the wheat low molecular weight glutenin promoter (Colot *et al.*  
25 (1987) *EMBO J* 6: 3559-3564).

A polyadenylation signal such as the NOS terminator may be used (Depicker et al. (1982) J. Mol Appl Genet 1: 499-512). A 3' UTR such as the modified sequence of TMV as described by Voss et al. (Mol. Breed. (1995) 1:39-50) may be used.

5

When introducing a chosen gene construct into a cell, certain considerations must be taken into account, well known to those skilled in the art. The nucleic acid to be inserted should be assembled within a construct which contains  
10 effective regulatory elements which will drive transcription. There must be available a method of transporting the construct into the cell. Once the construct is within the cell membrane, integration into the endogenous chromosomal material either will or will not occur. Finally, as far as  
15 plants are concerned the target cell type may be such that cells can be regenerated into whole plants, although as noted suspension cultures and callus cultures are within the present invention.

20 A plant cell or seed according to the present invention may be comprised in a plant or part (e.g. leaf, root, stem) or propagule thereof.

Plants which include a plant cell according to the invention  
25 are also provided, along with any part or propagule thereof, seed, selfed or hybrid progeny and descendants. A plant



according to the present invention may be one which does not breed true in one or more properties. Plant varieties may be excluded, particularly registrable plant varieties according to Plant Breeders' Rights. It is noted that a plant need not  
5 be considered a "plant variety" simply because it contains stably within its genome a transgene, introduced into a cell of the plant or an ancestor thereof.

In addition to a plant, the present invention provides any  
10 clone of such a plant, seed, selfed or hybrid progeny and descendants, and any part of any of these, such as cuttings, seed. The invention provides any plant propagule, that is any part which may be used in reproduction or propagation, sexual or asexual, including cuttings, seed and so on. Also  
15 encompassed by the invention is a plant which is a sexually or asexually propagated off-spring, clone or descendant of such a plant, or any part or propagule of said plant, off-spring, clone or descendant.

20 Plants transformed with an expression cassette containing the desired coding sequence may be produced by various techniques which are already known for the genetic manipulation of plants. DNA can be transformed into plant cells using any suitable technology, such as a disarmed Ti-plasmid vector  
25 carried by Agrobacterium exploiting its natural gene transfer ability (EP-A-270355, EP-A-0116718, NAR 12(22) 8711 - 87215

1984), particle or microprojectile bombardment (US 5100792, EP-A-444882, EP-A-434616) microinjection (WO 92/09696, WO 94/00583, EP 331083, EP 175966, Green et al. (1987) *Plant Tissue and Cell Culture*, Academic Press), electroporation (EP 5 290395, WO 8706614 Gelvin Debeyser - see attached) other forms of direct DNA uptake (DE 4005152, WO 9012096, US 4684611), liposome mediated DNA uptake (e.g. Freeman et al. *Plant Cell Physiol.* **29**: 1353 (1984)), or the vortexing method (e.g. Kindle, *PNAS U.S.A.* **87**: 1228 (1990d) (all incorporated 10 herein by reference). Physical methods for the transformation of plant cells are reviewed in Oard, 1991, *Biotech. Adv.* **9**: 1-11.

Agrobacterium transformation is widely used by those skilled 15 in the art to transform dicotyledonous species. Recently, there has been substantial progress towards the routine production of stable, fertile transgenic plants in almost all economically relevant monocot plants (Toriyama, et al. (1988) *Bio/Technology* **6**, 1072-1074; Zhang, et al. (1988) *Plant Cell* 20 *Rep.* **7**, 379-384; Zhang, et al. (1988) *Theor Appl Genet* **76**, 835-840; Shimamoto, et al. (1989) *Nature* **338**, 274-276; Datta, et al. (1990) *Bio/Technology* **8**, 736-740; Christou, et al. (1991) *Bio/Technology* **9**, 957-962; Peng, et al. (1991) International Rice Research Institute, Manila, Philippines 25 563-574; Cao, et al. (1992) *Plant Cell Rep.* **11**, 585-591; Li, et al. (1993) *Plant Cell Rep.* **12**, 250-255; Rathore, et al.

(1993) *Plant Molecular Biology* **21**, 871-884; Fromm, et al.  
(1990) *Bio/Technology* **8**, 833-839; Gordon-Kamm, et al. (1990)  
*Plant Cell* **2**, 603-618; D'Halluin, et al. (1992) *Plant Cell* **4**,  
1495-1505; Walters, et al. (1992) *Plant Molecular Biology* **18**,  
5 189-200; Koziel, et al. (1993) *Biotechnology* **11**, 194-200;  
Vasil, I. K. (1994) *Plant Molecular Biology* **25**, 925-937;  
Weeks, et al. (1993) *Plant Physiology* **102**, 1077-1084; Somers,  
et al. (1992) *Bio/Technology* **10**, 1589-1594; WO92/14828). In  
particular, *Agrobacterium* mediated transformation is now  
10 emerging also as an highly efficient alternative  
transformation method in monocots (Hiei et al. (1994) *The*  
*Plant Journal* **6**, 271-282).

The generation of fertile transgenic plants has been achieved  
15 in the cereals rice, maize, wheat, oat, and barley (reviewed  
in Shimamoto, K. (1994) *Current Opinion in Biotechnology* **5**,  
158-162.; Vasil, et al. (1992) *Bio/Technology* **10**, 667-674;  
Vain et al., 1995, *Biotechnology Advances* **13** (4): 653-671;  
Vasil, 1996, *Nature Biotechnology* **14** page 702) (all  
20 incorporated herein by reference).

Microprojectile bombardment, electroporation and direct DNA  
uptake are preferred where *Agrobacterium* is inefficient or  
ineffective. Alternatively, a combination of different  
25 techniques may be employed to enhance the efficiency of the

transformation process, eg bombardment with Agrobacterium coated microparticles (EP-A-486234) or microprojectile bombardment to induce wounding followed by co-cultivation with Agrobacterium (EP-A-486233).

5

Following transformation, a plant may be regenerated, e.g. from single cells, callus tissue, leaf discs, immature or mature embryos, as is standard in the art. Almost any plant can be entirely regenerated from cells, tissues and organs of the plant. Available techniques are reviewed in Vasil et al., *Cell Culture and Somatic Cell Genetics of Plants, Vol I, II and III, Laboratory Procedures and Their Applications*, Academic Press, 1984, and Weissbach and Weissbach, *Methods for Plant Molecular Biology*, Academic Press, 1989 (both  
10  
15 incorporated herein by reference) .

The particular choice of a transformation technology will be determined by its efficiency to transform certain plant species as well as the experience and preference of the person practising the invention with a particular methodology of choice. It will be apparent to the skilled person that the particular choice of a transformation system to introduce nucleic acid into plant cells is not essential to or a limitation of the invention, nor is the choice of technique  
20  
25 for plant regeneration.

A further aspect of the present invention provides a method of making a plant cell, preferably monocot, as disclosed involving introduction of a suitable vector including the relevant expression cassette into a plant cell and causing or  
5 allowing recombination between the vector and the plant cell genome to introduce the sequence of nucleotides into the genome. The invention extends to plant cells containing nucleic acid according to the invention as a result of introduction of the nucleic acid into an ancestor cell.

10

The term "heterologous" may be used to indicate that the gene/sequence of nucleotides in question have been introduced into said cells of the plant or an ancestor thereof, using genetic engineering, i.e. by human intervention. A  
15 transgenic plant cell, i.e. transgenic for the nucleic acid in question, may be provided. The transgene may be on an extra-genomic vector, such as a cow-pea mosaic viral vector, or incorporated, preferably stably, into the genome.

20 Following transformation of a plant cell, a plant may be regenerated from the cell or descendants thereof.

Further aspects of the present invention provide the use of an expression cassette with features disclosed herein (for  
25 example antibody encoding sequence or sequences fused to a mammalian ER retention signal, a peptide leader, and/or a

5'UTR as disclosed) in production of a transgenic plant cell and in production of a transgenic plant. Such a cell or plant is preferably monocot.

5 Transgenic plants in accordance with the present invention may be cultivated under conditions in which the desired product is produced in cells and/or seed of the plant. Cells producing the product may be in an edible part of the plant, such as leaves or fruit.

10

Following cultivation of plants, they, or parts thereof such as their leaves, seed or fruit, may be harvested and processed for isolation and/or purification of the product. Suitable techniques are available to those skilled in the art. The product may be used as desired, for instance in formulation of a composition including at least one additional component.

20

Seed may be stored, e.g. for at least six months.

25

Aspects and embodiments of the present invention will now be illustrated by way of experimental exemplification. Further aspects and embodiments of the present invention will be apparent to those skilled in the art.

*EXAMPLE 1*

The anti-CEA antibody T84.66 (US-A-5081235) has been used in clinical trials and has a proven potential for therapy and diagnosis.

5

The present inventors have successfully expressed the T84.66 antigen binding domain in the form of a scFv fragment (scFv84.66) in both rice and wheat. Various untranslated leader and leader peptide sequences were employed. See below

10 for details.

The single-chain fragments were either directed to the apoplast by means of an appropriate mammalian (murine) leader peptide sequence (e.g. construct CH84.66HP (Table 1 construct #1)) or retained in the endoplasmic reticulum by means of an ER retention signal (e.g. construct CH84.66KP (Table 1, construct #5)).

Functional expression of scFv able to bind its antigen was detected by ELISA in rice callus and leaves and in wheat leaves and seeds, both endosperm and embryo.

5/10 wheat plants transformed with CH84.66HP expressed the product in a range of 30-100 ng per gram of leaf material, with an average of 54 ng/g and a maximum of 100 ng/g.

19/30 wheat plants transformed with CH84.66KP expressed the product in a range of 50-700 ng/g, with an average of 243 ng/g and a maximum of 700 ng/g.

- 5 14/35 rice calli transformed with CH84.66HP expressed the product in a range of 30-300 ng/g. Four regenerated plants expressed the product in a range of 25-200 ng/g.

- 7/14 rice calli transformed with CH84.66KP expressed the  
10 product in a range of 70-3590 ng/g. Three regenerated plants expressed the product at 1500, 890 and 29000 ng/g leaf material, respectively.

- Transformation of rice with construct nr 7, containing the  
15 enhanced 35S promoter (2x35S), resulted in seven out of 11 lines expressing scFvT84.66 at levels between 500 and 27000 ng/g leaf tissue. Furthermore, western blot analysis of leaf extracts from selected rice lines transformed with this construct revealed that expressed scFvT84.66 was intact and  
20 had the predicted molecular weight.

Table 1 outlines the components of various expression cassettes (see below).

- 25 The ubiquitin promoter and the Nos terminator were used in constructs 1 to 6, the enhanced 35S promoter and terminator



were used in construct 7.

The results show that use of the ER retention signal enhances accumulation of the protein in wheat and rice plants, that  
5 the 5'UTR's are functional in wheat and rice, and that the mammalian leader peptide is functional in wheat and rice.

After six months of storage, the levels of functional, antigen-binding scFv 8466 were not significantly lower than  
10 at the time of harvest.

#### EXAMPLE 2

The anti-TMV antibody rAb 24 (heavy and light chain MBL accession numbers X67210 and X67211, respectively) is very  
15 well studied. See e.g. Voss et al. (1995) *Mol. Breed.* 1:39-50 (incorporated herein by reference).

This antibody has been expressed by the inventors in a single-chain Fv format (scFv24) in rice callus and plants.  
20 Particularly high amounts of the functional antibody fragment were detected by ELISA (Fischer et al. (1998) *Characterization and application of plant-derived recombinant antibodies*. In Cunningham C, Porter A (eds), "Methods in Biotechnology, Vol. 3: Recombinant Proteins from Plants: Production and Isolation of Clinically Useful Compounds"  
25 Methods in Biotechnology, Vol. 3, 129-142, Humana Press,

1997 (incorporated herein by reference)) in callus or rice containing a construct including a C-terminal ER retention signal.

- 5 A construct lacking any leader peptide sequence was introduced into rice. No expression was detectable by ELISA in callus tissue or leaves of these transformants.

A construct including the murine leader peptide and encoding  
10 scFv24 was used to transform rice and functional scFv was detected by ELISA in callus tissues and leaves. 3/4 rice calli expressed the product.

A further construct including the scFv24 coding sequence and  
15 a ER retention signal was expressed in transgenic rice. High levels of functional scFv were detected in callus. 12/25 calli expressed the product in a range of 300-42066 ng/g. One regenerated plant expressed the product at 8635 ng/g.

20 The results show that the mammalian light chain leader peptide is functional in rice and enhances protein levels as compared to cytosolic expression, and that the ER retention signal is functional in rice and enhances protein levels.

*EXAMPLE 3*

The full size chimeric (mouse/human) T84.66 antibody was successfully expressed in rice callus and plants

- 5 The genes for heavy and light chain of the antibody were located on two separate plasmids and introduced into plant cells via co-bombardment.

The enhanced 35S promoter was used in all constructs. The  
10 heavy and light chain were either both directed to the apoplast by means of an appropriate mammalian (murine) leader peptide sequence (Table 1, constructs 8 and 9) or, alternatively, the heavy chain was retained in the endoplasmic reticulum by means of an ER retention signal  
15 (Table 1, construct 10).

Functional expression of T84.66 able to bind its antigen was detected by ELISA in rice callus, leaves and seeds (Table 3). For a positive ELISA reaction, both the light chain and the  
20 heavy chain have to be expressed. If light and heavy chains are produced at different levels, the ELISA assay only indicates expression indicative of the lower expression level.

- 25 The results show that the genes for the heavy and light chain of a full size antibody can be stably transformed into a

plant cell on two separate plasmids. Functional antibody molecules are able to assemble in the plant cell if either both chains are targeted to the apoplast, or if one chain is retained in the ER.

5

*EXAMPLE 4*

The full size anti-TMV antibody rAb 24 was expressed in the apoplast of rice callus cells. The genes encoding the heavy and light chain were both driven by enhanced 35S promoter sequences and present on the same transformation vector.  
10 Seven out of 10 rice callus lines expressed functional (antigen binding) full size antibodies at levels between 100 and 50000ng/g.

15 The result shows that a functional anti-TMV antibody was produced in rice callus after introducing one plasmid containing the genes encoding heavy and light chain.

*EXAMPLE 5*

20 The anti-TMV antibody rAb 24 was expressed in rice callus and leaves in a Fab (construct 11), F(ab)<sub>2</sub> (construct 12) and bispecific single chain Fv format (construct 13). Various UTR and leader sequences were employed (constructs 11-13; Table 4). The enhanced 35S promoter and 35S terminator  
25 were used throughout.

Ten out of 18 rice callus lines transformed with construct 11 expressed the Fab24 fragment, directed to the apoplast, in a range of 30-5200 ng/g. A regenerated transgenic plant expressed the Fab fragment at 2500 ng/g leaf material.

5 Furthermore, western blot analysis of leaf extracts confirmed that expressed Fab 24 was intact and had the predicted molecular weight (double band at 28 kDa).

Three out of 5 rice callus lines containing construct 12 expressed functional (antigen binding) F(ab)<sub>2</sub> antibody fragments directed to the apoplast. The levels of F(ab)<sub>2</sub> measured were in the range of 100-29000 ng/g.

10

Six out of 8 rice callus lines containing construct nr 13 produced the bispecific single chain fragment of rAb24 in a range of 240 to 31000 ng/g. Two regenerated transgenic plants expressed the biscFv24 fragment in leaves at levels of 2100 or 1200 ng/g, respectively. In this case, an ER retention sequence was attached to the C-terminus of the antibody

15

20 fragment.

Rice callus lines containing construct 14, encoding the scFv24 fused to the coatprotein of TMV (tobacco mosaic virus), expressed the product at detectable levels. This was

25 determined by ELISAs based on the antigen binding capability of the scFv24.

These results show that various antigen binding fragments, such as Fab fragment, F(ab)<sub>2</sub> fragment, bispecific scFv and scFv fusion proteins can be expressed in callus and leaf tissue of transgenic rice lines.

5

#### EXAMPLE 6

Rice callus tissue was transformed with constructs containing the gene for scFv24 fused to various peptide signals for subcellular targeting. These targeting signals include the N-  
10 terminal chloroplast targeting signal of the structural gene for granule-bound starch synthase of potato (van der Leij et al., Mol Gen Gen (1991), 228: 240-248; incorporated herein by reference) and the N-terminal vacuolar targeting signal of strictosidine synthase from Catharanthus roseus (McKnight et  
15 al., Nucleic Acids Research (1990), 18, 4939; incorporated herein by reference).

Product expression was achieved at levels between 50 and 500 ng/g.

20

These results show that an antigen binding fragment, such as scFv, can be successful expressed in fusion with signal peptides for targeting to different subcellular compartments.

## EXAMPLE 7

Guy's 13 antibody is a secretory antibody (SigA) with specificity to the streptococcal antigen (SA) I/II cell surface adhesion protein of the oral pathogen *Streptococcus mutans* (Smith and Lehner (1989) *Oral Microbiol Immunol.* 4: 153). A secretory form of this antibody has been constructed and used in tobacco (Ma et al. (1995) *Science* 268: 716; incorporated herein by reference). The molecule consists of IgA dimers associated with the J-chain and the secretory component.

A chimeric mouse/human secretory antibody derived from Guy's 13 was expressed in transgenic rice lines. The four components, namely heavy chain, light chain, J-chain and secretory component, were encoded by four coding sequences, each driven by the maize ubiquitin promoter. The four cassettes were present on four separate plasmids and introduced into the plant cells by co-bombardment.

All coding sequences contained their natural leader peptides for secretion to the apoplast.

Fully assembled SigA was detected in several callus lines, up to a level of 800 ng/g. Fully assembled SigA was also detected in leaf material of a regenerated plant.

The result shows that complex antibodies, such as SigA, can be expressed in callus and leaves of rice following the introduction of the genes encoding the components on separate plasmids.

5

## *MATERIALS AND METHODS*

### Plasmids and Bacteria

#### *ScFv 84.66 plasmid construction*

A DNA fragment encoding the single-chain (scFv) protein  
10 derived from the anti-CEA antibody T84.66 was amplified by  
PCR using the construct pUC18-T84.66/212 (Wu et al., 1996  
*Immunotechnology* 2: 21-36; incorporated herein by reference))  
as a template, and specific primers introducing *Nco*I and *Sal*I  
restriction sites at the 5' and 3' ends respectively, for  
15 subcloning. The integrity of the scFvT84.66 gene was  
confirmed by DNA sequencing (ALF, Pharmacia).

The *Nco*I/*Sal*I amplified T84.66 fragment was subcloned into a  
pGEM3zf vector containing the 5' untranslated region of  
20 chalcone synthase (CHS 5' UTR) and the heavy chain leader  
peptide (muLPH\*) from the TMV virion-specific mAb24 (Voss et  
al., (1995) *Mol Breed* 1: 39-50). The muLPH\* sequence was  
codon optimised for plant expression according to Angenon et  
al. (*FEBS* (1990) 271: 144-146). Also included were either a  
25 KDEL motif or a His6 tag 3' to the T84.66 single-chain  
fragment as a C-terminal translation modification signal.



The whole cassette, containing either CHS 5' UTR-muLPH\*-T84.66-KDEL or CHS 5' UTR-muLPH\*-T84.66-His6 was recovered with *EcoRI* and *HindIII* digestion and subcloned into a pUC19 plasmid containing the maize ubiquitin 1 promoter, intron1 (US-A-5510474; (1990)) and the NOS termination sequence to give the final expression constructs. For co-transformation plasmid pAHC20 was used. This plasmid contains only the bar gene fused to the ubiquitin 1 promoter and intron 1 (Christensen and Quail (1996) *Transgen Res* 5: 213-218; incorporated herein by reference).

*scFv24 plasmid constructs for plant expression.*

The heavy and light chain cDNAs of rAb24 (EMBL accession numbers X67210 and X67211, respectively) were used for generation of scFv-cDNAs. The  $V_L$  and  $V_H$  fragments were amplified by PCR using domain-specific primers. For each domain one primer contained an overlapping sequence to form the  $V_L$  and  $V_H$  connecting linker (marked in italics) by splice overlap extension (SOE) PCR (Horton et al. "Engineering hybrid genes without the use of restriction enzymes; Gene splicing by overlap extension" Gene 77:61-68 (1989); incorporated herein by reference), and was used in conjunction with a primer containing either an *EcoRI* ( $V_L$ ) or a *SallI* ( $V_H$ ) restriction site (marked in bold).

25

The  $V_L$  domain was amplified using the forward primer P1-front:

5'-GCC**GAATTC**CATGGACGTCGAGCTGACCCAGTCT-3' (SEQ ID NO. 10),

and the reverse primer P2-back:

5'-CTTTCCGGAACCACTAGTAGAGCCTTTTATCTCCAGCTTGGT-3' (SEQ ID NO. 11).

5

The V<sub>H</sub> domain was amplified using the primers P3-front:

5'-GGTTCGGAAAGAGCTCTGAAGGTAAAGGTGAGGTCCAGCTGCAGCAG-3' (SEQ ID NO. 12) and P4-back:

5'-GCCTCTAGAC**GTCGACT**GCAGAGACAGTGACCAG-3' (SEQ ID NO. 13).

10

Individual V<sub>L</sub> and V<sub>H</sub> fragments were purified and assembled into a scFv fragment by SOE-PCR (Horton et al. (1989)) and subcloned into the *Eco*RI and *Sal*I restriction sites of a pUC18 derivative, containing a c-myc and His6 sequence. A

15 *Nde*I restriction site was introduced by PCR using the primer P5L24NL:

5'-GCACACCC**GAATTC**GGGCCCCGGG**CATATG**CAAATTGTTCTCACCCAGTCT-3'

(SEQ ID NO. 14), to enable cloning of the 5'-untranslated region of chalcone synthase (CHS 5'-UTR) as an *Eco*RI-*Nde*I

20 fragment.

The subsequent ligation of the *Eco*RI-*Xba*I fragment into the plant expression vector pSS (see below), containing an enhanced 35S promoter and the CaMV termination sequence,

25 resulted in the final construct pscFv24CW, which was used for

scFv expression in the cytosol. A second construct (pscFv24CM) was generated by exchanging the 5' *EcoRI*-*PstI* fragment of pscFv24CW with its corresponding region from the full-size light chain cDNA containing the CHS 5'-UTR and the original murine leader peptide sequence of the light chain cDNA of rAb24 to enable scFv secretion into the apoplast.

#### *Plant material*

Plants of *Triticum aestivum* L., cv Bobwhite, were grown in greenhouse and growthrooms at 15/12°C day/night temperature and 10 h photoperiod during the first 40 days, followed by maintenance at 21/18°C day/night temperature and 16 h photoperiod. Plants for insect bioassay were transferred to a heated glasshouse; day length was supplemented with artificial lighting to give a 16 h photoperiod, and temperature was maintained in the range 8-25°C.

#### *Target tissue and transformation*

Immature embryos were removed and cultured as described (Vasil et al. (1992) *Bio/Technology* 10: 667-674). After 6 to 7 days, particle bombardment was performed using standard conditions. Thirty to seventy micrograms of coated gold particles/shot were delivered to the target tissue which was incubated on medium containing high osmoticum (0.2 M mannitol and 0.2 M sorbitol) for 5-6 hours prior to and 10-16 hours after bombardment. Plasmids containing the unselected gene

and the plasmid containing the bar gene were mixed for co-transformation at a molar ratio of 3:2 and precipitated onto gold particles (Christou et al., 1991 Bio/Technology 9: 957-962; incorporated herein by reference).

5

Bombarded callus was selected on medium containing phosphinothricin, as described elsewhere (Altpeter et al., 1996, Plant Cell Rep 16: 12-17; incorporated herein by reference).

10

#### *PAT assays*

PAT activity was assayed using leaf tissue as described before transferring the plants to soil (Vasil et al., (1992) Bio/Technology 10: 667-674).

15

#### *Production of Monoclonal Antibody and CEA antigen*

The pPIC9K yeast expression vector containing the CEA/NA3 domain and the mAb84.66 was used. The CEA/NA3 protein was expressed in *Pichia pastoris* strain GS115 (InVitrogen) and  
20 purified from the fermentation broth using Ni-NTA affinity chromatography.

The hybridoma cell line T84.66 (Wagener et al., 1983 *Journal of Immunology* 130: 2308-2315; incorporated herein by  
25 reference) was grown in RPMI 1640 (Biochrom) containing 10% fetal calf serum (Biochrom), 25 mM NaHCO<sub>3</sub>, 1 mM L-glutamine,

50  $\mu$ M 2-mercaptoethanol, 24 mM sodium bicarbonate, 50 IU penicillin and 50  $\mu$ g streptomycin per ml (Gibco) and maintained at 37°C in a humidified incubator with 7% CO<sub>2</sub>.

Immunoglobulins from culture supernatants were subjected to  
5 affinity chromatography on protein-A HC (BioProcessing). The purity of the mAb preparation was analysed by SDS-PAGE (Laemmli 1970). The presence of CEA-specific antibodies was ascertained by ELISA.

#### 10 *Protein extraction and ELISA*

Extraction of total soluble proteins from leaves and seeds was performed as described by Fischer et al. (1998)

*(Characterization and application of plant-derived recombinant antibodies. In Cunningham C, Porter A (eds),*

15 "Methods in Biotechnology, Vol. 3: Recombinant Proteins from Plants: Production and Isolation of Clinically Useful Compounds", Methods in Biotechnology, Vol. 3, 129-142, Humana Press Inc., 1997).

20 Functional T84.66 single-chain antibody was measured in an enzyme linked immunosorbent assay (ELISA) by competition with the full-size murine T84.66 monoclonal antibody. Microtitre plates were coated with 50 ng CEA/NA3 in bicarbonate buffer and blocked with 150  $\mu$ l bovine serum albumin (1.0% in saline  
25 buffer (0.85% NaCl, pH7.2)). Serial dilutions of plant extracts were made using extracts from non-infiltrated

control leaves, and 100  $\mu$ l of each diluted sample, also containing 2.5 ng full-size murine T84.66 antibody was transferred to the CEA/NA3 coated and blocked ELISA plate. Alkaline phosphatase-conjugated Fc specific goat anti-mouse IgG (100 $\mu$ l of a 1:5000 dilution; Jackson ImmunoResearch) was added to each well, and plates were then developed for up to 1 h at 37°C with 100  $\mu$ l AP substrate (1 mg ml<sup>-1</sup> p-nitrophenylphosphate, Sigma, in substrate buffer (0.1M Diethanolamine, 1 mM MgCl<sub>2</sub>, pH9.8) before reading the absorption at 405 nm using a Spectra Max 340 spectrophotometer (Molecular Devices).

#### *Southern and Northern blot*

DNA was prepared from leaf tissue according to Dellaporta et al., (1984) *Maize DNA miniprep*. In Malmberg R, Messing J, Sussex I (eds), "Molecular biology of plants. A laboratory course manual", pp36-37. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; incorporated herein by reference. 15 $\mu$ g aliquots of DNA were digested with appropriate restriction endonucleases and subjected to electrophoresis on 0.9% agarose gels. Transfer to nylon membranes and hybridisation were carried out according to standard procedures (Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY.)

25

Total RNA was isolated from leaves of transgenic wheat

plants, subjected to agarose gel electrophoresis (15 µg per lane) and blotted to a nitrocellulose membrane. <sup>32</sup>P-labelled hybridisation probes comprising the coding region of the transgene were prepared using the random primer labelling kit  
5 (GIBCO-BRL).

### Results

*Production and characterisation of transgenic wheat plants*  
Production of transgenic wheat plants by bombardment of  
10 immature embryos has been described previously (Altpeter et al., 1997). The gene coding for scFv84.66 and the *bar* gene, as a selectable marker, were co-transformed into wheat on two separate plasmids. Nine to ten weeks after bombardment, regenerated plantlets were tested for phosphinothricin  
15 acetyltransferase (PAT) expression. Forty independent transgenic lines were identified. Thirty lines had been co-bombarded with plasmid pCH84.66KP encoding the scFv antibody with an added KDEL-signal for retention in the ER. The remaining ten lines had been co-bombarded with pCH84.66HP  
20 containing the His-tag instead of the KDEL sequence.

Southern blot analysis was carried out on a representative sample of fifteen primary transformants and confirmed the presence of the *bar* gene in all lines tested. Hybridisation  
25 with a probe for the scFv coding sequence revealed the integration of the gene in 11 lines with a co-transformation

frequency of ca. 80%. The transgene integration patterns were clearly unique for each line and the complexity of integration varied within the range expected for plants generated via direct gene transfer.

5

*Expression of scFv in leaves*

Extracts of soluble proteins from transgenic leaves were assayed for scFv presence and activity by ELISA. Eighteen out of 27 plants transformed with construct pCH84.66KP showed  
10 production levels of up to 700 ng functional active scFv84.66 per g leaf tissue (range: 50-700 ng). The maximum expression level detected in plants containing construct pCH84.66HP was 100 ng per g leaf tissue (range 30-100 ng).

15 *Expression of scFv in seeds*

Mature seeds from the best expressing plants were harvested and extracts of soluble proteins were used for ELISA. Up to 1.5  $\mu$ g scFv per g seed were determined. These levels of expression exceeded the levels measured in leaves.

20

*Construction of cT84.66 heavy and light chain cDNAs*

Splice overlap extension (SOE) PCR was used to obtain full-size mouse/human chimeric T84.66 light and heavy chain cDNAs, by in frame fusion of the variable VL and VH domains of the  
25 mouse mAb T84.66 to the human kappa and IgG1 constant domains of the B72.3 mouse/human chimeric antibody DNAs (Primus et



al. (1990) *Cancer Immunol Immunother* **31**, 349-57; incorporated herein by reference). The human constant domains were amplified from plasmids chiB72.3L and chiB72.3H using the following primers: 5'-CTG GAA ATA AAA ACT GTG GCT GCA CCA  
5 TCT-3' (chiB72.3L-I) (SEQ ID NO. 15), 5'-GCC AAG CTT TTT GCA  
AAG ATT CAC-3' (chiB72.3L-II) (SEQ ID NO. 16), 5'-ACC GTC TCC  
TCA GCC TCC ACC AAG GGC CCA-3' (chiB72.3H-I) (SEQ ID NO. 17),  
and 5'-GCC AAG CTT GGA TCC TTG GAG GGG CCC AGG-3' (chiB72.3H)  
(SEQ ID NO. 18).

10

The mouse variable domains were amplified from plasmids T84.66L2 (light chain) and T84.66H2 (heavy chain) using the primers: 5'-GGC GAA TTC ATG GAG ACA GAC ACA CTC-3' (T84.66L-I) (SEQ ID NO. 19), 5'-AGC CAC AGT TTT TAT TTC CAG CTT GGT  
15 CCC-3' (T84.66L) (SEQ ID NO. 20), 5'-GGC GAA TTC ATG AAA TGC  
AGC TGG GTT-3' (T84.66H) (SEQ ID NO. 21), 5'-GGT GGA GGC TGA  
GGA GAC GGT GAC TGA GGT-3' (T84.66H) (SEQ ID NO. 22).

Chimeric T84.66 light and heavy chain cDNAs obtained by SOE  
20 PCR were cloned as EcoRI/HindIII fragments into pUC18, to  
give the constructs pUC18-"Light" and pUC18-"Heavy",  
respectively. All cDNA sequences were confirmed by nucleotide  
sequencing.

25 *Construction of full-size cT84.66 plant expression plasmids.*  
pGEM-3zf was used for cloning the 5'UTR from the omega leader

region of tobacco mosaic virus (TMV) (Schmitz et al. (1996) *Nucleic Acids Res* **24**, 257-63), followed by one of the two plant codon optimised leader peptides derived either from the heavy chain (LPH) or from the light chain (LPL) of the murine mAb24 (Voss et al. (1995) *Molecular Breeding* **1**, 39-50), and for cloning the KDEL ER-retention signal sequence, and the 3'UTR from TMV. Chimeric light chain was digested with NcoI/SalI and inserted downstream from the 5' omega region of TMV and the LPL; chimeric heavy chain was inserted the same way (construct 9), or downstream from the 5' omega region of TMV and the LPH, and upstream from the KDEL sequence (construct 10). The expression cassettes were cloned between the enhanced 35S promoter and the cauliflower mosaic virus termination region utilising the EcoRI and XbaI restriction sites of the pSS plant expression vector (Voss et al. (1995) *Molecular Breeding* **1**, 39-50).

*Construction of bispecific single chain Fv 24 plant expression vectors*

To combine scFv24 and the CBHI linker with scFv29 in a bispecific single chain antibody, a cassette arrangement was chosen with restriction sites at the 5' and 3' ends of the two scFv and linker sequences.

First, the scFv29 was subcloned into the EcoRI and SalI restriction sites of a pUC18 derivate, containing a His6

sequence (pUC18-scFv29-his). The plasmid pML2 containing the cDNA of the CBHI-linker was used in conjunction with the forward primer CBH-CLA 5'-GCG GAA TTC GTA ATC GAT CCC GGG GGT AAC CGC GGT ACC-3' (SEQ ID NO. 23) and backward primer CBH-  
5 MOD 5'-GCG GAC GTC GCT ATG AGA CTG GGT GGG CCC-3' (SEQ ID NO. 24) to introduce an EcoRI and ClaI (5' end) or an AatII (3' end) restriction site(s) by PCR. The EcoRI and AatII restricted PCR fragment was subcloned into pUC18-scFv29-his (CBHI-scFv29-his). EcoRI and NcoI restriction sites were  
10 integrated at the 5' end of scFv24 (Zimmermann et al. (1998) *Molecular Breeding* 4, 369-379; incorporated herein by reference) by PCR using the primer SCA25 5'-G CGG AAT TCG GCC ACC ATG GCC CAA ATT GTT CTC ACC CAG TCT-3' (SEQ ID NO. 25) and a 3' ClaI site using the primer SCA26 5'-GCG ATC GAT TGC  
15 AGA GAC AGT GAC CAG AGT-3' (SEQ ID NO. 26). Cloning of the EcoRI-ClaI fragment upstream of the CBHI linker in the vector pUC18-scFv29-his gave the biscFv2429 construct pUC18-biscFv2429.

20 For targeting biscFv2429 to different plant cell compartments, the 5' EcoRI-StuI fragment of pUC18-biscFv2429, containing the 5' end of scFv24, was exchanged with its corresponding region from pscFv24CM (Zimmermann et al. (1998) *Molecular Breeding* 4, 369-379) containing the 5' untranslated  
25 region of the chalcon synthase (CHS 5'-UT) (Voss et al. (1995) *Molecular Breeding* 1, 39-50) and the original mouse leader

sequence of the light chain cDNA. The C-terminal His6 sequence of biscFv2429 was replaced with the ER retention signal KDEL, which was introduced by PCR using the primer KDEL: 5'-ACG CTC TAG AGC TCA TCT TTC TCA GAT CCA CGA GAA CCT  
5 CCA CCT CCG TCG ACT GCA GAG ACA GTG ACC AGA GTC CC-3' (SEQ ID NO. 27) to generate pUC18-biscFv2429-KDEL. The subsequent ligation of the EcoRI-XbaI fragment into the plant expression vector pSS (Voss et al. (1995) *Molecular Breeding* 1, 39-50), containing an enhanced 35S promoter and the CaMV termination  
10 sequence, resulted in the final expression construct biscFv2429-KDEL (Table 4, construct 13), which was used for biscFv2429 expression in the endoplasmic reticulum.

*Construction of the plant transformation vector encoding a*  
15 *scFv24-coatprotein fusion*

The gene fusion partner coat protein (CP) from TMV was amplified by PCR. cDNA was amplified from a cDNA clone from TMV. The forward primers introduced a NcoI restriction site (5' end) and the backward primers a C-terminal (Gly4Ser)2  
20 linker sequence and an AatII restriction site (3' end). The following forward and backward primer were used for PCR amplification:

CP-for 5'-ACT GCG CCA TGG CTT ACA GTA TCA CT-3' (SEQ ID NO. 28),  
25 CP-back 5'-CCG TCA GAC GTC AGA ACC TCC ACC TCC ACT TCC GCC GCC TCC AGT TGC AGG ACC AGA GGT CCA AAC CAA ACC-3' (SEQ ID

NO. 29).

The 5'-NcoI and 3'-AatII restricted PCR fragments were subcloned into a pUC18 derivative containing the TMV specific scFv24 (Zimmermann et al. (1998) *Molecular Breeding* 4: 369-379) flanked by the 5' untranslated region (omega-sequence) and 3' untranslated region (Pw sequence) from TMV (Schmitzet al. (1996) *Nucleic Acids Res* 24: 257-263; Gallie et al., (1994) *Gene* 142: 159-165).

10

A C-terminal KDEL-sequence was added to scFv24 by PCR using the backward primer KDEL-back 5'-CCC TCA CTC GAG TTT AGA GCT CAT CTT TCT CAG ATC CAC GAG CGG CCG CAG AAC CTC CAC CTC CGT CGA CTG CAG AGA CAG TGA CCA G-3' (SEQ ID NO. 30). The subsequent ligation of the EcoRI-AscI fragments into the plant expression vector pSS, containing an double enhanced 35S promoter (Voss et al., 1995), resulted in the final expression construct CP-scFv24K.

20 *Construction of pscFv24-VTS:*

The plant codon optimized (for rice, wheat and tobacco) N-terminal vacuolar targeting signal of strictosidine synthase from *Catharanthus roseus* (McKnight et al., 1990) was added to the scFv24 by PCR using the forward primers VTS5': 5'-GCC GAA TTC ATA TGG CAA ACT TCT CTG AAT CTA AGT CCA TGA TGG CAG TTT TCT TCA TGT TTT TCC TTC TTC TCC TTT C -3' (SEQ ID NO. 31) and

25

VTS3': 5'-ATG TTT TTC CTT CTT CTC CTT TCA TCT AGC TCT TCA AGC  
TCT TCA TCT TCC ATG GGA CAA ATT GTT CTC ACC CAG TCC C-3' (SEQ  
ID NO. 32), which introduce a 5' EcoRI and NdeI and a NcoI  
restriction site at the 3' end of the vacuolar targeting  
5 sequence. scFv24CW (Zimmermann et al., 1998) was used as  
template and a pUC specific oligo as a backward primer. The  
NdeI and HindIII restricted PCR fragment was subcloned into  
scFv24CW. The scFv24, cmc and his6 containing NcoI/HindIII  
fragment was replaced by an identical but already sequenced  
10 fragment. The subsequent ligation of the EcoRI/SalI fragment  
into the plant expression vector pSS containing a C-terminal  
c-myc and his6 sequence resulted in the final expression  
construct pscFv24-VTS.

15 *Construction of pscFv24-CTS:*

The plant codon optimized (for rice, wheat and tobacco) N-  
terminal chloroplast targeting signal of the structural gene  
for granule-bound starch synthase of potato (van der Leij et  
al., Mol Gen Gen 1991, 228: 240-248) was added to the scFv24  
20 by PCR using four forward primers: PrimCTS1: 5'-GCC GAA TTC  
ATA TGG CAT CTA TCA CTG CTT CTC ACC ACT TTG TGT CTA GGT CTC  
AAA CTT CTC TTG ACA CC-3' (SEQ ID NO. 33), PrimCTS2: 5'-GGT  
CTC AAA CTT CTC TTG ACA CCA AAT CTA CCT TGT CTC AGA TCG GAC  
TCA GGA ACC ATA CTC TTA CTC AC-3' (SEQ ID NO. 34), PrimCTS3:  
25 5'-TCA GGA ACC ATA CTC TTA CTC ACA ATG GTT TGA GGG CTG TTA  
ACA AGC TCG ATG GTC TCC AAT CTA GAA C-3' (SEQ ID NO. 35),

PrimCTS4: 5'-CTC GAT GGT CTC CAA TCT AGG ACT AAT ACT AAG GTC  
ACC CCT AAG ATG GCA TCT AGG ACT GAG ACC AAG AGG C-3' (SEQ ID  
NO. 36); and PrimCTS5: 5'-GCA TCT AGG ACT GAG ACC AAG AGG CCA  
GGA TGC TCT GCT ACC ATT GTT TGC GCC ATG GGA CAA ATT GTT CTC  
5 ACC CAG TCT C-3' (SEQ ID NO. 37), which introduce 5' EcoRI  
and 5' NdeI restriction sites and a NcoI restriction site at  
the 3' end of the chloroplast targeting sequence. scFv24CW  
(Zimmermann et al., 1998) was used as template and a pUC  
specific oligo as a backward primer. The amplified PCR  
10 product was digested with NdeI and HindIII and subcloned into  
scFv24CW. The scFv24, c-myc and his6 containing NcoI/HindIII  
fragment was replaced by an identical but already sequenced  
fragment. The construct was digested with EcoRI and SalI and  
the EcoRI/SalI fragment containing the scFv sequence was  
15 subsequently ligated into the plant expression vector pSS  
containing a C-terminal c-myc and his6 sequence resulted in  
the final expression construct pscFv24-CTS.

#### *Construction of plasmids encoding the SigA components*

20

A human/mouse hybrid kappa chain was assembled as follows.

An XhoI/HindIII fragment containing the Guy's light variable  
region, and a HindIII/EcoRI fragment containing the human  
25 kappa constant region were ligated together with the native  
mouse heavy chain leader sequence (muLPH) into a pUC19

plasmid containing the maize ubiquitin 1 promoter, intron 1 and the NOS termination sequence to give the final expression construct.

- 5 A KpnI/EcoRI fragment containing the human J chain was ligated into a pUC19 plasmid containing the maize ubiquitin 1 promoter, intron 1 and the NOS termination sequence.

*Construction of Fab24 and F(ab)<sub>2</sub> 24*

10

Splice overlap extension (SOE) PCR was used to obtain Fab fragments.

Fusion oligonucleotides 5'- C TGT CCT CCA TGA GCT CAG CAC  
15 CCA CAA AAC -3' (31 mer) (SEQ ID NO. 38) and 5'- GTG CTG AGC  
TCA TGG AGG ACA GGG GTT GAT -3' (30 mer) (SEQ ID NO. 39) were  
used for the SOE of the mouse IgG2b hinge domain and of the  
3'-UT of mouse IgG2b in order to obtain Fab-fragments. The  
final SOE product contains one S-S-bridge (1. cys of the  
20 hinge) to the mouse kappa light chain. The second cysteine  
residue was converted to a TGA stop codon. This  
oligonucleotide represents the (+)strand and can be used as a  
backward primer in a PCR to amplify the mouse 3'-UT of IgG2b.  
The overlap to the mouse hinge domain is 22 bp.

25

To obtain F(ab)<sub>2</sub> fragments, fusion oligonucleotides



5'-A TGC AAG GAG TGA GCT CAG CAC CCA CAA AGC-3' (31 mer) (SEQ ID NO. 40) and 5'-TG CTG AGC TCA CTC CTT GCA TGG AGG ACA G-3' (30 mer) (SEQ ID NO. 41) were used for the SOE of the mouse IgG2b hinge domain and of the 3'-UT of mouse IgG2b in order to obtain F(ab')<sub>2</sub> fragments. The final SOE product contains two S-S-bridges (1. cys of the hinge to the mouse kappa light chain and the second to the IgG2b heavy chain). The third cys residue was converted to a TGA stop codon. This oligonucleotide represents the (+) strand and can be used as a backward primer in a PCR to amplify the mouse IgG2b in order to obtain mouse F(ab')<sub>2</sub>. The overlap to the mouse hinge domain is 21 bp.

The modified cDNA-Fab and F(ab)<sub>2</sub> fragments were fused to the chalcone synthase (CHS) 5'UTR and subcloned into the plant expression vector pSS, containing the enhanced 35S promoter and CaMV termination signal.

#### *J Chain*

A Kpn I/EcoR I fragment containing the human J chain was ligated to pMON530. Cloning was confirmed by restriction digest and by PCR analysis.

#### *Secretory Component*

25

Full length native human secretory component was assembled

from three sequenced fragments, HuSC2, HuSC3a and the 5' portion of HuSC (up to the first Acc I site). First, the plasmid containing HuSC was cut with Kpn and religated, to remove the Acc I and EcoR I sites in the vector polylinker. This was confirmed by restriction digest. Plasmids containing HuSC2 and HuSC3 were digested with Xma I and EcoR I, ligated, and selected on chloramphenicol (only one of the two original plasmids was chloramphenicol resistant). Fusion of HuSC2 and HuSC3a was confirmed by restriction digest. An Acc I/EcoR I fragment from the HuSC2/3a clone was used to replace the corresponding fragment in the HuSC clone. The assembled clone was thus made of fully sequenced subfragments, contained Kpn I and Nco I sites at the 5' end, an EcoR I site at the 3' end, and no internal Kpn I sites. Correct assembly was confirmed by restriction digests on. The re-assembled Kpn I/EcoR I fragment was ligated to pMON530. Clones were screened by restriction digests. Correct assembly was confirmed by additional restriction digests.

20

*Gamma/Alpha Heavy Chain*

A human/mouse hybrid heavy chain was assembled as follows. Plasmids containing the IgG1 C<sub>H</sub>1-C<sub>H</sub>2 domains (pHUG) and the Guy's 13 heavy variable region (pGuyHV-2) were both cut with  
25 Apa I. A fragment containing the IgG1 C<sub>H</sub>1-C<sub>H</sub>2 domains was

ligated to the Apa I cut pGuyHV-2. Clones were screened by restriction digest. The resulting hybrid was called pGUY/HUG.

5 Clones pHuA2 and pHuA3, containing fragments HuA2 and HuA3 respectively, were cut with BspE I and Sac II. The insert fragment released from pHuA3 was ligated to the linearized pHuA2, fusing the C<sub>H</sub>2-C<sub>H</sub>3 encoding domains together. Assembly was confirmed by restriction digest. The resulting hybrid  
10 was called pHuA2/3.

Plasmid pHuA2/3 was cut with Hind III and Sma I. Plasmid pGUY/HUG was cut with Hind III and Hinc II. The Hua2/3 fragment was ligated to the linearized pGUY/HUG. Correct  
15 assembly was confirmed by restriction digests. The resulting clones contain the complete hybrid (glycosylated) heavy chain. The entire cassette was cut out as a Kpn I/Eco RI fragment and cloned into pMON530.

## 20 DISCUSSION

The results show that the 5'UTR's, the petunia chalcon synthase and viral omega sequences, are functional in wheat and rice, also the TMV 3'UTR. The mammalian leader peptide sequences, both heavy and light chain, are shown by the  
25 results to be functional in cereal callus, leaves and seeds. Use of the ER retention signal produced a higher level of

antibody than in the apoplasm. Within the constructs carrying KDEL, CL84.66KP (Construct 4) and OL84.66KP (Construct 6) led to better production levels than analogous constructs containing the murine heavy chain leader peptide, 5 providing indication of advantageous use of leader peptide influencing production level of the expression product in rice.

With the scFv24, expressed in rice callus and plants, 10 pscFv24, lacking any 5' leader peptide or 3' signal sequence, did not provide scFv24 at a level detectable using ELISA. A construct containing the gene for scFv including the murine leader peptide (of the light chain) gave detectable levels of scFv24 in transgenic callus lines, although below 200ng/g. 15 The construct additionally containing a 3' KDEL sequence yielded the highest levels of scFv, up to 42066 ng/g, range 300-42066 ng/g.

TABLE 1

Constructs containing forms of T84.66.

	<u>Nr</u>	<u>Promoter</u>	<u>cDNA construct</u>	<u>ter</u>
5				
	1	ubiquitin	5' UTR(CHS)-muLPH*-scFv84.66-His6-3' UTR(PW-TMV) abbreviation: CH84.66HP	NOS
	2	ubiquitin	5' UTR(CHS)-muLPL*-scFv84.66-His6-3' UTR(PW-TMV) abbreviation: CL84.66HP	NOS
10	3	ubiquitin	5' UTR(Ome)-muLPH*-scFv84.66-KDEL-3' UTR(PW-TMV) abbreviation: OH84.66KP	NOS
	4	ubiquitin	5' UTR(CHS)-muLPL*-scFv84.66-KDEL-3' UTR(PW-TMV) abbreviation: CL84.66KP	NOS
	5	ubiquitin	5' UTR(CHS)-muLPH*-scFv84.66-KDEL-3' UTR(PW-TMV) abbreviation: CH84.66KP	NOS
15	6	ubiquitin	5' UTR(Ome)-muLPL*-scFv84.66-KDEL-3' UTR(PW-TMV) abbreviation: OL84.66KP	NOS
	7	2x35S	5' UTR(CHS)-muLPH*-scFv84.66-KDEL-3' UTR(PW-TMV)	35S
	8	2x35S	5' UTR(Ome)-muLPL*-muVL-huCL-3' UTR(PW-TMV)	35S
20	9	2x35S	5' UTR(Ome)-muLPH*-muVH-huCH-3' UTR(PW-TMV)	35S
	10	2x35S	5' UTR(Ome)-muLPH*-muVH-huCH-KDEL-3' UTR(PW-TMV)	35S
	UTR	untranslated region		
	CHS	5'UTR of chalcon synthetase		
25	Ome	Omega sequence of TMV (5'-translational enhancer)		
	muLP	murine leader peptide		
	LPH*	heavy chain leader peptide of $\alpha$ -TMV mAB24, codon optimised for tobacco, pea + wheat		
	LPL*	light chain leader peptide of $\alpha$ -TMV mAb24, codon		

		optimised for tobacco, pea + wheat
	scFv	single chain Fv fragment
	84.66	$\alpha$ -CEA antibody T84.66 (binds to A3 domain with high affinity)
5	His6	histidine 6 for Ni-NTA based affinity chromatography
	KDEL	C-terminal KDEL motif to enable ER-retention (leads to increased protein accumulation)
	stop	stop codon
	PW	pseudoknot region of TMV-wildtype 3'UTR (potential
10		transcriptional and translational enhancer)
	TMV	tobacco mosaic virus
	ubiquitin	ubiquitin 1 promoter and intron from maize
	2x35S	enhanced 35S promoter from cauliflower mosaic virus
	NOS	terminator from the Nopaline synthase gene of
15		Agrobacterium
	35S	terminator from cauliflower mosaic virus
	muVL	murine light chain variable region
	muVH	murine heavy chain variable region
	huCL	human light chain constant region
20	huCH	human heavy chain constant regions

TABLE 2

Results of experiments using the cassettes shown in Table 1 to express the scFv84.66 in rice callus and leaves. The ubiquitin promoter and the NOS pA were used throughout.

<u>Expression cassette</u>		<u>callus</u>	<u>leaf</u>	<u>seed</u>
		mean ng/g	mean ng/g	mean ng/g
10	construct 1	129	59	110
	construct 2	n.d.	61	n.d.
	construct 3	762	1250	n.d.
	construct 4	1663	3030	2800
	construct 5	758	10460	10050
15	construct 6	1229	1460	n.d.
	construct 7	n.d.	8930	n.d.

TABLE 3

Functional expression of T84.66 able to bind its antigen detected by ELISA in rice callus, leaves and seeds.

5	<u>Expression cassette</u>	<u>callus</u>	<u>leaf</u>	<u>seed</u>
		(ng/g)	(ng/g)	(ng/g)
	Construct 8+9	100-250	250	200-300
10	Construct 8+10	100-300	280	200-390

TABLE 4

Constructs containing forms of rAb24

15	<u>Nr</u>	<u>Promoter</u>	<u>cDNA construct</u>	<u>ter</u>
	11	2x35S	5'UTR(CHS)-muLPL-VL24-CL-3'UTR	35S
		2x35S	5'UTR(CHS)-muLPH-VH24-CH1-3'UTR	35S
			(The two cassettes are in tandem)	
20	12	2x35S	5'UTR(CHS)-muLPL-VH24-CL-3'UTR	35S
		2x35s	5'UTR(CHS)-muLPH-VH24-CH1(2cys)-3'UTR	35S
			(The two cassettes are in tandem)	
	13	2x35S	5'UTR(CHS)-muLPL-VL24-VH24-VL29-VH29-KDEL	35S
	14	2x35S	5'UTR(Ome)-muLPL-CP-scFv24-KDEL-3'UTR(PW-TMV)	35S

25

CP coat protein of TMV



CLAIMS:

1. A monocotyledonous plant cell or seed containing a mammalian polypeptide produced by expression within the cell or  
5 seed from an expression cassette comprising a coding sequence for the polypeptide, in which plant cell or seed there is an additional feature selected from the group consisting of:
- (a) the polypeptide is expressed fused to an endoplasmic reticulum (ER) retention signal;
  - 10 (b) the coding sequence for the polypeptide is preceded in the expression cassette by 5' untranslated leader sequence (5'UTR);
  - (c) the polypeptide is expressed fused to a leader peptide;
  - (d) any combination of two of (a), (b) and (c); and
  - 15 (e) a combination of (a), (b) and (c).
2. A plant cell or seed according to claim 1 wherein the retention signal is a peptide with the amino acid sequence KDEL (SEQ. ID NO. 2) or HDEL (SEQ. ID NO. 4).
- 20
3. A plant cell or seed according to claim 1 wherein the 5'UTR is a chalcone synthase 5'UTR.
4. A plant cell or seed according to claim 3 in which the  
25 expression cassette comprises the following 5'UTR sequence:
- 5'-GAATTCACAACACAAATCAGATTTATAGAGAGATTTA

TAAAAAAAAAAAAACATATG-3' (SEQ. ID NO. 7).

5. A plant cell or seed according to claim 1 wherein the 5'UTR is a TMV omega gene 5'UTR.

5

6. A plant cell or seed according to claim 5 wherein the 5'UTR has the following RNA sequence:

5'-GUAUUUUUACAACAUAUACCAACAACAACAACAACAAC

AUUACAUAUACUAUUUACAAGGACCAUGG-3' (SEQ ID NO. 9).

10

7. A plant cell or seed according to claim 1 wherein the leader peptide is a mammalian leader peptide.

8. A plant cell or seed according to claim 7 wherein the  
15 leader peptide is an immunoglobulin light or heavy chain leader peptide.

9. A plant cell or seed according to claim 1 wherein the leader peptide is a vacuole targeting signal

20

10. A plant cell or seed according to claim 1 wherein the leader peptide is a chloroplast targeting signal

11. A plant cell or seed according to claim 1 wherein the  
25 leader peptide causes transport into protein bodies.

12. A plant cell or seed according to claim 1 which is a rice cell or seed.

13. A plant cell or seed according to claim 1 which is a wheat  
5 cell or seed.

14. A cereal plant cell or seed containing a mammalian protein produced by expression within the cell or seed from an expression cassette comprising a coding sequence for the  
10 protein.

15. A plant cell or seed according to claim 14 that is rice or wheat.

15 16. A plant cell or seed according to claim 1 wherein an antibody molecule is produced within the cell or seed.

17. A plant cell or seed according to claim 16 wherein the antibody molecule is a single chain Fv antibody fragment.

20

18. A plant cell or seed according to claim 16 wherein the antibody molecule is a two-chain or multi-chain complex which comprises a plurality of polypeptides and is selected from the group consisting of Fv, Fab, F(ab)<sub>2</sub>, diabody, dimeric scFv,  
25 whole antibody and four-chain secretory antibody.

19. A plant cell or seed according to claim 18 wherein each polypeptide in said plurality of polypeptides is expressed from a separate expression vector within the cell or seed.
- 5 20. A plant cell or seed according to claim 19 wherein the antibody molecule is a four-chain secretory antibody and each of the four chains is expressed from a separate expression vector within the cell or seed.
- 10 21. A plant cell or seed according to claim 1 wherein the cell or seed is actively producing the polypeptide.
22. A suspension culture or callus culture comprising a plant cell according to 21.
- 15 23. A plant cell or seed according to claim 1 comprised in a plant or plant part.
24. A plant or plant part comprising a plant cell or seed  
20 according to claim 1.
25. A method of making a monocotyledonous plant cell comprising an expression cassette as claimed in claim 1, the method comprising:
- 25 (i) introducing into a plant cell a nucleic acid suitable for transformation of a plant cell and comprising the expression

cassette, and

(ii) causing or allowing recombination between the nucleic acid and the plant cell genome to introduce the expression cassette into the genome.

5

26. A method according to claim 25 wherein the plant cell is transformed with a plurality of vectors, each of the plurality of vectors comprising an expression cassette comprising a coding sequence for a different polypeptide of a multi-chain complex  
10 which comprises a plurality of polypeptides, wherein on production of the polypeptides by expression within the plant cell or descendants thereof the multi-chain complex is formed.

27. A method according to claim 26 wherein the plant cell is  
15 transformed with four vectors, each vector encoding a different polypeptide chain of a four-chain secretory antibody, wherein on production of the polypeptides by expression within the plant cell or descendants thereof the secretory antibody is formed.

20 28. A method according to claim 25 further comprising growing plant cells in plant cell culture to produce the mammalian polypeptide.

29. A method according to claim 28 further comprising isolating  
25 and/or purifying the mammalian polypeptide from the plant cell culture.

30. A method of making a plant, the method comprising:

- (i) making plant cells according to claim 25, and
- (ii) regenerating a plant from said plant cells or descendants thereof.

5

31. A method according to claim 30 further comprising growing plants to produce the mammalian polypeptide.

32. A method according to claim 31 further comprising isolating  
10 and/or purifying the mammalian polypeptide from the plants or parts of the plants.

33. Use of an expression construct comprising a coding sequence for a mammalian polypeptide, in which expression cassette there  
15 is an additional feature selected from the group consisting of:

- (a) a sequence coding for an endoplasmic reticulum (ER) retention signal and providing for expression of the polypeptide fused to the endoplasmic reticulum retention signal;

- (b) the coding sequence for the polypeptide is preceded in  
20 the expression cassette by 5' untranslated leader sequence (5'UTR);

- (c) a sequence coding for a leader peptide and providing for expression of the polypeptide fused to a leader peptide;

- (d) any combination of two of (a), (b) and (c); and

- 25 (e) a combination of (a), (b) and (c);

for production of transgenic plant cells which produce the

polypeptide.

34. Use of an expression construct comprising a coding sequence for a mammalian polypeptide, in which expression cassette there is an additional feature selected from the group consisting of:

(a) a sequence coding for an endoplasmic reticulum (ER) retention signal and providing for expression of the polypeptide fused to the endoplasmic reticulum retention signal;

(b) the coding sequence for the polypeptide is preceded in the expression cassette by 5' untranslated leader sequence (5'UTR);

(c) a sequence coding for a leader peptide and providing for expression of the polypeptide fused to a leader peptide;

(d) any combination of two of (a), (b) and (c); and

(e) a combination of (a), (b) and (c);

for production of transgenic plants comprising cells or seed which produce the polypeptide.

**FIGURE 1**

<b>Promoter</b>	<b>5'UTR</b>	<b>Leader peptide</b>	<b>Gene of interest</b>	<b>sig</b>	<b>3'UTR</b>	<b>pA</b>
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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Paul CHRISTOU; Eva STROGER; Rainer FISCHER; Carmen MARTIN-VAQUERO; Stefan SCHILLBERG; Julian K-C MA
- (ii) TITLE OF INVENTION: METHODS AND MEANS FOR EXPRESSION OF MAMMALIAN POLYPEPTIDES IN MONOCOTYLEDONOUS PLANTS
- (iii) NUMBER OF SEQUENCES: 41
- (iv) CORRESPONDENCE ADDRESS:
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  - (F) ZIP: 10103
- (v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: Diskette, 3.25 inch, 1.44mb
  - (B) COMPUTER: IBM PS/2
  - (C) OPERATING SYSTEM: PC-DOS
  - (D) SOFTWARE: Wordperfect
- (vi) CURRENT APPLICATION DATA:
- (A) APPLICATION NUMBER: To Be Assigned
  - (B) FILING DATE: Concurrently Herewith
  - (C) CLASSIFICATION: 435
- (vii) PRIOR APPLICATION DATA:
- (A) APPLICATION NUMBER: 60/089,322
  - (B) FILING DATE: June 15, 1998
- (viii) ATTORNEY/AGENT INFORMATION:
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## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AAAGATGAGC TC

12

## (2) INFORMATION FOR SEQ ID NO: 2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 4
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Lys Asp Glu Leu

## (2) INFORMATION FOR SEQ ID NO: 3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 12
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CATGATGAGC TC

12

## (2) INFORMATION FOR SEQ ID NO: 4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 4
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

His Asp Glu Leu

## (2) INFORMATION FOR SEQ ID NO: 5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Gly Gly Gly Gly Ser  
5

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 4
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Arg Gly Ser Glu

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 57
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GAATTCACAA CACAAATCAG ATTTATAGAG AGATTTATAA AAAAAAAAAA ACATATG

57

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 71
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GUAUUUUUAC AACAAUUAAC AACACAACA AACACAAAC AACAUUACAA UUACUAUUUA

60

CAAUACAAT G

71

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 70
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GUAUUUUUAC AACAAUACC AACACAACA ACAACAACAA CAUUACAAU ACUAAUUACA 60  
AGGACCAUGG 70

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GCCGAATTCC ATGGACGTCG AGCTGACCCA GTCT 34

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 42
- (B) TYPE: nucleic acids
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CTTTCCGGAA CCACTAGTAG AGCCTTTTAT CTCCAGCTTG GT 42

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 48
- (B) TYPE: nucleic acids
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GGTTCCGGAA AGAGCTCTGA AGGTAAAGGT GAGGTCCAGC TGCAGCAG 48

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

GCCTCTAGAC GTCGACTGCA GAGACAGTGA CCAG 34

## (2) INFORMATION FOR SEQ ID NO: 14:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

GCACACCCGA ATTCGGGCCC GGGCATATGC AAATTGTTCT CACCCAGTCT

50

## (2) INFORMATION FOR SEQ ID NO: 15:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CTGGAAATAA AAACGTGTGGC TGCACCATCT

30

## (2) INFORMATION FOR SEQ ID NO: 16:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

GCCAAGCTTT TTGCAAAGAT TCAC

24

## (2) INFORMATION FOR SEQ ID NO: 17:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

ACCGTCTCCT CAGCCTCCAC CAAGGGCCCA

30

## (2) INFORMATION FOR SEQ ID NO: 18:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

GCCAAGCTTG GATCCTTGGA GGGGCCCAGG

30

## (2) INFORMATION FOR SEQ ID NO: 19:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

GGCGAATTCA TGGAGACAGA CAACTC

27

## (2) INFORMATION FOR SEQ ID NO: 20:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

AGCCACAGTT TTTATTTCCA GCTTGGTCCC

30

## (2) INFORMATION FOR SEQ ID NO: 21:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

GGCGAATTCA TGAAATGCAG CTGGGTT

27

## (2) INFORMATION FOR SEQ ID NO: 22:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

GGTGGAGGCT GAGGAGACGG TGACTGAGGT

30

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 39
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

GCGGAATTCG TAATCGATCC CGGGGGTAAC CGCGGTACC

39

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

GCGGACGTCG CTATGAGACT GGGTGGGCCC

30

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 43
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

GCGGAATTCG GCCACCATGG CCCAAATTGT TCTACCCAG TCT

43

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

GCGATCGATT GCAGAGACAG TGACCAGAGT

30

## (2) INFORMATION FOR SEQ ID NO: 27:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 77
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

ACGCTCTAGA GCTCATCTTT CTCAGATCCA CGAGAACCTC CACCTCCGTC GACTGCAGAG 60  
ACAGTGACCA GAGTCCC 77

## (2) INFORMATION FOR SEQ ID NO: 28:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

ACTGCGCCAT GGCTTACAGT ATCACT 26

## (2) INFORMATION FOR SEQ ID NO: 29:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 72
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

CCGTCAGACG TCAGAACCTC CACCTCCACT TCCGCCGCCT CCAGTTGCAG GACCAGAGGT 60  
CCAAACCAAA CC 72

## (2) INFORMATION FOR SEQ ID NO: 30:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 88
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

CCCTCACTCG AGTTTAGAGC TCATCTTTCT CAGATCCACG AGCGGCCGCA GAACCTCCAC 60  
CTCCGTCGAC TGCAGAGACA GTGACCAG 88



## (2) INFORMATION FOR SEQ ID NO: 31:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 79
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

GCCGAATTCA TATGGCAAAC TTCTCTGAAT CTAAGTCCAT GATGGCAGTT TTCTTCATGT 60  
TTTTCCTTCT TCTCCTTTC 79

## (2) INFORMATION FOR SEQ ID NO: 32:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 79
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

ATGTTTTTCC TTCTTCTCCT TTCATCTAGC TCTTCAAGCT CTTTCATCTTC CATGGGACAA 60  
ATTGTTCTCA CCCAGTCCC 79

## (2) INFORMATION FOR SEQ ID NO: 33:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 71
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

GCCGAATTCA TATGGCATCT ATCACTGCTT CTCACCACTT TGTGTCTAGG TCTCAAACCTT 60  
CTCTTGACAC C 71

## (2) INFORMATION FOR SEQ ID NO: 34:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 71
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

GGTCTCAAAC TTCTCTTGAC ACCAAATCTA CCTTGTCTCA GATCGGACTC AGGAACCATA 60  
CTCTTACTCA C 71

## (2) INFORMATION FOR SEQ ID NO: 35:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 70
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

TCAGGAACCA TACTCTTACT CACAATGGTT TGAGGGCTGT TAACAAGCTC GATGGTCTCC 60  
AATCTAGAAC 70

## (2) INFORMATION FOR SEQ ID NO: 36:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 73
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

CTCGATGGTC TCCAATCTAG GACTAATACT AAGGTCACCC CTAAGATGGC ATCTAGGACT 60  
GAGACCAAGA GGC 73

## (2) INFORMATION FOR SEQ ID NO: 37:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 82
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

GCATCTAGGA CTGAGACCAA GAGGCCAGGA TGCTCTGCTA CCATTGTTTG CGCCATGGGA 60  
CAAATTGTTC TCACCCAGTC TC 82

## (2) INFORMATION FOR SEQ ID NO: 38:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:

CTGTCCTCCA TGAGCTCAGC ACCCACAAAA C 31

## (2) INFORMATION FOR SEQ ID NO: 39:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:

GTGCTGAGCT CATGGAGGAC AGGGGTTGAT

30

## (2) INFORMATION FOR SEQ ID NO: 40:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40:

ATGCAAGGAG TGAGCTCAGC ACCCACAAAG C

31

## (2) INFORMATION FOR SEQ ID NO: 41:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 41:

TGCTGAGCTC ACTCCTTGCA TGGAGGACAG

30

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